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Previously we have shown that ATM phosphorylates SMC1 to regulate radiosensitivity. This project is to investigate whether interfering the ATM-SMC1 DNA damage signaling will increase the sensitivity of breast tumor cells to radiotherapy and chemotherapy. To achieve this goal, we have synthesized two small peptides containing the original amino acid sequence of SMC1 around Ser957. We have found that the peptide that has the wild-type SMC1 sequence possess the inhibitory ability on ATM kinase activity *in vitro*. We have also designed TAT-fusion peptides that can deliver the SMC1 peptide into cells. We have shown that the TAT-fusion peptides: 1) can be internalized into cells in a time- and dose-dependent manner; 2) can abrogate radiation-induced S-phase checkpoint; and 3) have minimal cytotoxicity to breast cancer cells in the absence of DNA damage. These insights are providing a basis for developing strategies for increasing selective tumor cell cytotoxicity after chemotherapy and radiotherapy. Future experiments will be focusing on testing the radio- and chemo-sensitization effect of the fusion peptides.

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Introduction:

Recent studies have revealed that cellular sensitivity to DNA damage agents is controlled by the Ataxia-Telangiectasia-Mutated (ATM) protein kinase and its signal to the Structural Maintenance of Chromosome protein one (SMC1)(6,7,11). ATM phosphorylation of SMC1 at two serine sites is required for limiting the amount of radiation sensitivity. We hypothesized that targeting the ATM-SMC1 pathway may lead to a novel approach for developing sensitizers to therapeutic interventions for breast cancer. This hypothesis has been tested in this proposal by studying the effect of synthetic peptides that aim to block the in vivo phosphorylation events on breast cancer cellular response to DNA damage. The long term goal of this project is to develop the concept for utilizing novel approach to targeting DNA damage pathway in order to sensitize breast tumor to radiotherapy and chemotherapy.

Body:

A. Development of a small peptide to inhibit ATM phosphorylation of SMC1 *in vitro*. (SOW Task 1a and 1b)

Since ATM phosphorylation of SMC1 at two serine sites is required for limiting the amount of radiation sensitivity, we hypothesized that small molecule that can modulate ATM-SMC1 phosphorylation may function as radiosensitizers. Since a general consensus target motif has been defined for ATM--it phosphorylates a serine or threonine residue only if it is followed by a glutamine (the "SQ/TQ motif) (5,8), these molecules could consist of the SQ sequence of SMC1 (at Ser957 and Ser966), presumably targeting ATM phosphorylation of SMC1. To test this hypothesis, we synthesized two small peptides to test the inhibitory effect on ATM kinase activity in an *in vitro* kinase assay (**Fig 1A**). One peptide contains the original amino acid sequence of SMC1 around Ser957 (peptide 2), and the other originates from the sequence except two serine and the glutamine residues were replaced (peptide 1). Flag-tagged ATM was immunoprecipitated, followed by an *in vitro* kinase assay using GST-SMC1 (amino acid 951-964) as a substrate in the presence of ^{32}P-ATP. Our data shows that peptide 1 does not effect ATM phosphorylation on SMC1, while peptide 2 totally abrogates the phosphorylation signal (**Fig 1B**). This observation suggests that small peptides containing the amino acid sequence of ATM phosphorylation may have an inhibitory effect on ATM substrate recognition and phosphorylation; therefore they can be tested as *in vivo* inhibitors and radiosensitizers.

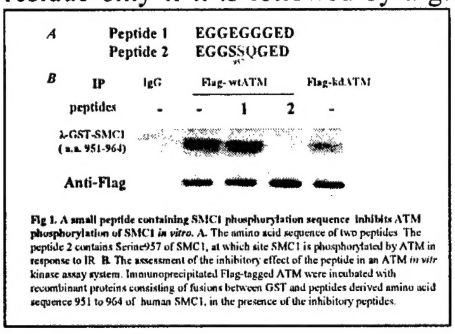

Fig 1. A small peptide containing SMC1 phosphorylation sequence inhibits ATM phosphorylation of SMC1 *in vitro*. A. The amino acid sequence of two peptides. The peptide 2 contains Serine957 of SMC1, at which site SMC1 is phosphorylated by ATM in response to IR. B. The assessment of the inhibitory effect of the peptide in an ATM *in vitr* kinase assay system. Immunoprecipitated Flag-tagged ATM were incubated with recombinant proteins consisting of fusions between GST and peptides derived amino acid sequence 951 to 964 of human SMC1, in the presence of the inhibitory peptides.

B. Determination of peptide internalization (SOW Task 1a, c and d)

Since small peptides can transport through cellular membrane only when the molecules are very small—typically less than 600 Daltons (9), the proposed peptides need to link to a protein transduction domain. Recent reports have suggested that conjugation of peptides, proteins and antisense to short highly basic peptides, such as the human immunodeficiency virus (HIV) TAT, results in their rapid translocation into cells (2). TAT-mediated delivery requires a short 11 amino acid region of the TAT protein (1,3,4,9). This region corresponds to amino acid 45-57 of TAT (**YGRKKRRQRRR**) and

has a high net positive charge at physiological pH with nine out of 11 of its amino acids being either arginine or lysine. The 11-amino-acid TAT sequence carries full-length proteins or small peptides into cells in a rapid, concentration-dependent fashion that appears to be independent of receptors and transporters and instead is through targeting the lipid bi-layer component of cell membrane. Therefore, in principal, all mammalian cell type should be susceptible to the internalization (9). Based on above information, we synthesized three fusion peptides. These peptides are composed of two functional domains, one an interference domain and the other a targeting domain (**Fig 2A**). The N-terminus of the peptides was linked with biotin for visualization. For the interference domain, the SMC1 phosphorylation sequence (amino acid sequence around Serines 957 and 966) was included. Mutant sequence (serine to alanine substitution, which abrogates the phosphorylation) was generated as a negative control (**Fig 2B**). For the targeting domain, we utilized the TAT sequence.

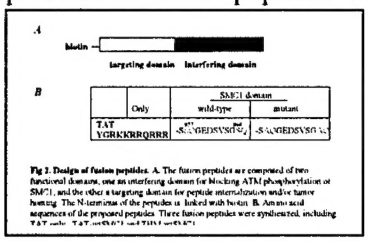

Fig 2. Design of fusion peptides. A. The fusion peptides are composed of two functional domains, one an interfering domain for blocking ATM phosphorylation of SMC1, and the other a targeting domain for peptide internalization and/or tumor homing. The N-terminus of the peptides is linked with biotin. B. Amino acid sequences of the proposed peptides. Three fusion peptides were synthesized, including

Our first set of experiments to characterize the peptides was to test the internalization effect of the fusion peptides. MCF-7 cells were incubated with 10μM of the peptides for 30 minutes and the immunofluoresence microscopic assay was used to assess the localization of the peptides. Since the peptides are linked with biotin on their N-terminus, an FITC-conjugated anti-streptavidin antibody, which recognizes the tetrameric protein that can bind to biotin, was used to visualize the peptides (**Figure 3**). Our data confirmed that the fusion peptides localize within cytoplasmic and nuclear portions of the cell.

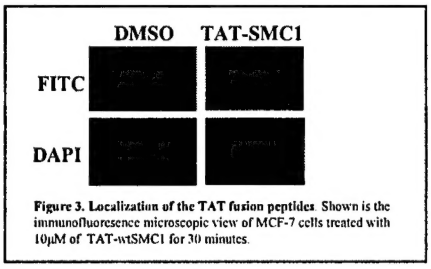

Figure 3. Localization of the TAT fusion peptides. Shown is the immunofluoresence microscopic view of MCF-7 cells treated with 10μM of TAT-wtSMC1 for 30 minutes.

C. Determine the cytotoxicity effect of the peptides (SOW Task 1e)

A promising radiosensitizer should be non-toxic to cells, but sensitize only tumor cells to radiation. To test the toxicity of the peptides, MCF-7, a well characterized breast cancer cell line on which we have studied the radiation sensitivity and cell cycle checkpoints was utilized. Cell viability was measured using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. We find that the TAT peptides have minimal cytotoxicity to MCF-7 cells as shown in **Figure 4** that there is no decrease of cell survival after 24 hours treatment of the peptides. The effect of the peptides on cell cycle distribution was also investigated. Cells treated with peptides were harvested, fixed in 70% of ethanol, and stained with propidium iodide, followed by a flow cytometric analysis. No change of basal cell cycle distribution is detected (data not shown).

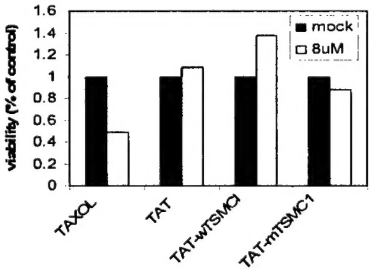

Figure 4. The TAT-fusion peptides possess minimal cytotoxicity to breast tumor cells. MCF-7 cells were treated with different doses of TAT-peptides or Taxol for three hours. After incubation, cell viability was quantified using a standard MTT assay. Shown is cell survival in the presence of 8uM TAT-peptides.

D. Investigate the inhibitory effect of the peptides on IR-induced S-phase checkpoint activation (SOW Task 2b).

Since ATM phosphorylation of SMC1 regulates IR-induced S-phase arrest, we then investigated the inhibitory effect of the TAT-SMC1 peptides on IR-induced S arrest in MCF-7 cells. To measure the S-phase arrest, the RDS assay (10) was utilized. We find that in the presence of TAT-wtSMC1 peptide, MCF-7 cells display the Radio-Resistance DNA synthesis phenotype, indicative of abrogation of the S-phase checkpoint **(Figure 5)**. However, the TAT only and TAT-mtSMC1 peptides have no effect on IR-induced S-phase checkpoint. These observations suggest that the wild-type SMC1 phosphorylation sequence specifically interferes with endogenous SMC1 phosphorylation after IR; therefore it is quite likely that this peptide will function as a powerful radiosensitizer.

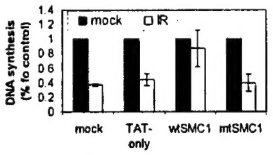

Fig 5. Exposure to the TAT-wtSMC1 peptide abrogates IR-induced S-phase checkpoint. To assess the S-phase checkpoint, MCF-7 cells were treated with 10μM of either TAT only, TAT-wtSMC1, or TAT-mtSMC1. 30 min after exposure to 10 Gy of ionizing radiation, replicative DNA synthesis was measured. *Columns*, averages of at least triplicate samples; *bars*, SE.

Key research accomplishments:

1. We have found that a small peptide that contains the SMC1 phosphorylation sequence has an inhibitory effect on ATM activity; and
2. This peptide, when linked with the TAT sequence, can block radiation-induced S-phase checkpoint.

Reportable outcomes:

We will present our observations in major scientific meetings, such as the Annual Meeting of American Association of Cancer Research.

Conclusions

The major focus of this project is to develop the concept for utilizing novel approach to targeting DNA damage pathway in order to sensitize breast tumor to radiotherapy and chemotherapy. We have found that it is possible to use small peptides containing SMC1 phosphorylation sequence to interfere with ATM-mediated SMC1 phosphorylation. These peptides can then be tested as powerful sensitizers. This project may yield new therapeutic agents to improve the sensitivity of human breast cancers to radiotherapy and chemotherapy. This approach may also generate specific inhibitors to DNA damage pathways for basic breast cancer research.

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Appendices

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